Central Nervous System Damage

The invention relates to materials and methods useful in the treatment of, or in the development of treatments for, CNS damage, e.g. spinal cord injury or stroke.

Background

Most tissues in the body, such as skin, liver and peripheral nerve, have a remarkable ability to repair themselves after injury. By contrast, the central nervous system (CNS) - including the brain and the spinal cord - has little innate capacity for repair. When axonal connections are damaged in the adult brain or spinal cord, they show an extremely limited ability to regenerate, even though axons can grow and regenerate efficiently in the embryonic CNS and in the adult peripheral nervous system. Factors that account for the inability of CNS axons to regenerate can be grouped in two categories: intrinsic properties of CNS neurons that may make them incapable of regeneration; and extrinsic factors in the CNS environment that are inhibitory to axonal elongation.

The idea that factors in the CNS environment can prevent regeneration dates back to the early 20th century. Ramon y Cajal observed that the inability of adult CNS neurons to extend axonal processes could be overcome by giving them the permissive environment of a peripheral nerve. Then, about 20 years ago, David and Aguayo showed that retinal neurons could form long projections in peripheral nerve grafts. Later, Schwab discovered that dorsal root ganglion neurons in culture extend their axons across Schwann cells but avoid oligodendrocytes and the fatty myelin sheath (Schwab et al 1993).

These results show that failure to regenerate is not purely an intrinsic deficit of CNS neurons, and inhibitory factors in the CNS environment also play an important role. These inhibitory factors are mainly located in the glial scar that forms at the region of injury and by the myelin that ensheaths axons in the white matter tracks.

Following CNS injury, the central area of necrosis is infiltrated by glia and other non-neuronal cells, and a fibrous scar forms. Axons do not extend through the scar and their growth appears to be inhibited by it. Molecular components that may contribute to this inhibitory activity include the extracellular matrix glycoprotein tenascin-R (TN-R) and the myelin-associated neurite outgrowth inhibitors myelin-associated glycoprotein (MAG) and Nogo.

TN-R

TN-R has been implicated in a variety of cell-matrix interactions involved in the molecular control of axon guidance and neural cell migration during development and regeneration of CNS (reviewed by Erickson, 1993; Chiquet-Ehrismann et al., 1994; Pesheva et al., 2000; 2001). TN-R is the smallest member of the tenascin family and is composed of four structural motifs: a cysteine-rich segment at the N-terminus is followed by 4.5 EGF-like repeats. This region is followed by 9 consecutive fibronectin type III-like domains and at the C-terminus TN-R is related to the beta- and- gamma-chains of fibrinogen.

TN-R is expressed predominantly by oligodendrocytes during the onset and early phases of myelin formation, and remains expressed by some oligodendrocytes in the adult. TN-R is

also expressed in some neurons and interneurons in the spinal cord, retina, cerebellum, and hippocampus (Fuss et al., 1991; 1993). TN-R co-localizes with other glial-derived molecules (i.e. myelin-associated glycoprotein and a phosphacan-related molecule) at high density in the nodes of Ranvier of CNS myelinated axons (Xiao et al., 1997: Yang et al, 1999).

TN-R can inhibit or promote neurite outgrowth, depending on the neuronal cell type and the environment in which it is presented. When TN-R was offered, acting as a sharp substrate boundary, dorsal root ganglion (DRG), cerebellum and retinal ganglion neuron growth cones avoided growing on these molecules, but were not induced to collapse. On the other hand, when TN-R was offered in a mixture with laminin (which strongly promotes growth of embryonic and adult axons) as a uniform substrate, DRG growth cones displayed a collapsed morphology and were able to advance at a faster rate than on laminin alone.

Using several monoclonal antibodies binding to distinct epitopes on the tenascin molecule, epidermal growth factor-like (EGF-L) repeats and fibronectin type III homologous repeats 4-5 were identified to be responsible for growth cone repulsion.

In vitro, outgrowth of embryonic and adult retinal ganglion cell axons from mouse retinal explants is significantly reduced on homogeneous substrates of tenascin-R or a bacterially expressed tenascin-R fragment comprising the EGF-L domain. When both molecules are presented, acting as a sharp substrate boundary, regrowing adult axons do not cross into the territory containing tenascin-R or EGF-L. All in vitro experiments were done in the presence of

laminin, suggesting that tenascin-R and EGF-L actively inhibit axonal growth. Neurites and growth cones were repelled from areas coated with fragments containing the EGF-L (the amino-terminal cysteine-rich domain plus the EGF-like repeats), FN (fibronectin) 1-2, FN3-5 and FG (fibrinogen) domains of TN-R, and EGF-L prevents neurite outgrowth of hippocampal neurons.

TN-R also induces axonal defasciculation in vitro through the EGF-L domain (Taylor et al., 1993; Xiao et al., 1996, 1997, 1998; Becker et al, 1999; Becker et al, 2000).

After 3-acetylpyridine-induced lesion of the olivocerebellar system of the adult rat, the density of cells containing TN-R transcripts increased significantly in the inferior olivary nucleus and in the white matter of the cerebellar cortex. Immunohistochemical investigations confirmed these observations at the protein level.

After a spinal cord mechanical lesion of rat, TN-R mRNA was also upregulated (Wintergerst at al, 1997; Deckner et al, 2000).

These findings suggested that the continued overexpression of TN-R in the injured CNS may contribute to the failure of adult axonal regeneration in vivo.

Tenascin-R is a member of the tenascin family, which play important roles in cell interactions in the developing nervous system, such as neuronal migration, neuritogenesis, and neuronal regeneration. Tenascin-R is expressed predominantly by oligodendrocytes during the onset and early phases of myelin formation, and remain expressed by some oligodendrocytes in the adult (Pesheva et. al., 1989; Fuss

et al., 1991,1993; Wintergerst et. al., 1993; Ajemian A. et. al., 1994). TN-R is a multi-functional molecule (Lochter and Schachner, 1993; Pesheva et. al., 1993; Taylor et. al., 1993; Xiao et. al., 1996, 1997,1998), and it has indicated that it is an inhibitory component of myelin extraction. Xiao have found that, the EGF-L domain of TN-R can inhibit the neurite outgrowth.

MAG

MAG is a transmembrane protein of the immunoglobulin superfamily expressed by myelinating glial cells of the central and peripheral nervous systems, where MAG represents 1 and 0.1% of the total myelin proteins, respectively (Heape at al, 1999). MAG is a potent inhibitor of axonal regeneration and also, depending on the age and type of neuron, can promote axonal growth. MAG inhibits neurite outgrowth of retinal, superior cervical ganglion, spinal, and hippocampal and dorsal root ganglion (DRG) neurons of all postnatal ages, but can enhance neurite outgrowth of embryonic spinal cord neurons and newborn DRG neurons (DeBellard at al, 1996; Turnley et al, 1998; Shen et al, 1998; Yang et al, 1999).

MAG can also induce growth cone collapse. 60% of axonal growth cones of postnatal day 1 hippocampal neurons collapsed when they encountered coated recombinant MAG (rMAG). Such collapse was not observed with denatured rMAG (Li et al, 1996). Soluble dMAG (a proteolytic fragment of the extracellular domain of MAG, which is released in abundance from myelin and found in vivo) and chimeric MAG-Fc can potently inhibit neurite outgrowth from P6 DRG neurons. This inhibition was blocked when a MAG monoclonal antibody was included.

These results indicate that soluble dMAG detected in vivo could contribute to the lack of regeneration in the mammalian CNS after injury (Tang et al, 1997; 2001).

MAG has two recognition sites for neurons, the sialic acid binding site at R118 and a distinct inhibition site which is absent from the first three Ig domains (Tang et al, 1997).

MAG is a well characterized member of the immunoglobulin gene superfamily, and it exerts a robust inhibitory effect on neurite outgrowth from young cerebellar neurons and adult dorsal ganglion (DRG) neurons (Mukhopadhyay et al., 1994). MAG is a membrane protein with 626 amino acids. It has been reported that soluble MAG, which consists of the extracellular domain, has an inhibitory effect on neurite outgrowth (Mckerracher et. al., 1994). The extracellular domain of MAG consists of five Ig-like domains, and it is demonstrated that the first two Ig-like domains are important for the interaction between MAG and neuronal membrane, while the other three Ig-like domains might be involved in the inhibitory effects (Collins et al., 1997).

Nogo

Nogo is a high molecular weight integral membrane protein that localizes to CNS myelin, but not PNS myelin. Nogo has three isoforms, named Nogo-A, -B and -C, which are generated by alternative splicing. NI-250 and NI-35 were first identified and named as the 2 isoforms of Nogo; it has now been established that NI-250 is Nogo-A and NI-35 is Nogo-B. Nogo is expressed by oligodendrocytes in white matter of the CNS and is found in the inner and outer leaflets of myelin and in the endoplasmic reticulum.

In vitro characterization of Nogo has demonstrated its function as a potent inhibitor of axon elongation. In vivo neutralization of Nogo activity results in enhanced axonal regeneration and functional recovery following CNS injury as well as increased plasticity in uninjured CNS fibers. The monoclonal antibody mAb IN-1 was shown to promote long-distance regeneration and functional recovery in vivo when applied to spinal cord-injured adult rat (Chen et al, 2000; GrandPre et al; Merkler et al).

These findings suggest that Nogo may be a major contributor to the nonpermissive nature of the CNS environment. Two distinct inhibitory domains of Nogo have been identified: an intracellular amino-terminal domain (NogoN) of Nogo A and a short 66 residue region (Nogo-66) located between two hydrophobic domains of the three isoforms, Nogo-A, Nogo-B and Nogo-C (Chen et al, 2000; GrandPre et al, 2000; Fournier et al , 2001; Filbin, 2003). These domains are illustrated in Science, Vol 297 (5584), 16 Aug 2002, p. 1132-1134.

Nogo-A is expressed by oligodendrocytes but not by Schwann cells. It can inhibit axonal extension and collapses dorsal root ganglion growth cones (GrandPre et. al., 2000). The neurite outgrowth inhibitory activity of Nogo can be neutralized by monoclonal antibody IN-1, which allows axonal regeneration and functional recovery after spinal cord injury (Chen et. al., 2000). Nogo is a membrane protein with 1163 amino acids. The C-terminal tail contains two hydrophobic transmembrane domains separated by a 66-residue hydrophilic extracellular domain. This 66-residue extracellular domain can inhibit axon outgrowth (Fournier et. al., 2001).

Huang et al. (1999) discloses a therapeutic vaccine approach to stimulate axonal regeneration in the adult spinal cord.

It is an object of the invention to provide materials and methods useful in the treatment of, or in the development of treatments for, CNS damage, e.g. spinal cord injury, by overcoming the inhibitory effects of myelin on axonal regeneration.

Summary of Invention

In a first general aspect, the inventor proposes using molecules which interact with the inhibitory domains of major myelin proteins as an adjunct treatment for CNS damage, and for the development of further treatments.

In a second general aspect, the inventor proposes immunizing subjects with the inhibitory domains of major myelin proteins as an adjunct treatment for CNS damage.

It may appear impractical to immunize subjects against myelin antigens, as this approach may induce autoimmune demyelinating disease, leading to unacceptable side effects. Also, some myelin antibodies promote remyelination (Rodriguez et al., 1987), which is not of benefit during axonal regeneration. The inventor's approach is, however, to base the vaccine on the specific inhibitory portions of major myelin proteins, instead of whole myelin proteins. Antibodies raised against the inhibitory portions will block the inhibitory effect of myelin on axonal regeneration.

<u>First aspect</u>

Accordingly, the invention provides a peptide, the amino acid sequence of which consists of an amino acid sequence selected from the group consisting of:

YLTQPQS (SEQ ID NO. 1);
GSLPHSL (SEQ ID NO. 2);
TQLFPPQ (SEQ ID NO. 3);
HSIPDNI (SEQ ID NO. 4);
HHMPHDK (SEQ ID NO. 5);
YTTPPSP (SEQ ID NO. 6); and
QLPLMPR (SEQ ID NO. 7).

These sequences each represent the deduced amino acid sequences of several peptides that have been identified by phage display as being capable of binding to one or more of the neuronal growth inhibitory molecules Nogo (specifically, the Nogo-66 domain), MAG and TN-R (specifically, TNR-EGFL).

SEQ ID NO. 1 represents the sequence of 43 identical peptides capable of binding to Nogo-66, and also the sequence of 19 identical peptides capable of binding to MAG.

SEQ ID NO. 2 represents the sequence of 8 identical peptides capable of binding to Nogo-66.

SEQ ID NO. 3 represents the sequence of 18 identical peptides capable of binding to TNR-EGFL.

SEQ ID NO. 4 represents the sequence of 3 identical peptides capable of binding to TNR-EGFL.

SEQ ID NO. 5 represents the sequence of 1 peptide capable of binding to TNR-EGFL.

SEQ ID NO. 6 represents the sequence of 1 peptide capable of binding to TNR-EGFL.

SEQ ID NO. 7 represents the sequence of 5 identical peptides capable of binding to MAG.

Of these, SEQ ID NO. 1 has been shown by phage binding to block the inhibitory effects of Nogo-66 and MAG on neuronal cell adhesion in an in vitro assay. Similarly, SEQ ID NO. 3 has been shown to block the inhibitory effect of TNR-EGFL. A preferred peptide therefore consists of SEQ ID NO. 1; another preferred peptide consists of SEQ ID NO. 3.

The invention further provides a peptide up to 60 amino acids in length comprising an amino acid sequence selected from the group consisting of:

YLTQPQS (SEQ ID NO. 1);
GSLPHSL (SEQ ID NO. 2);
TQLFPPQ (SEQ ID NO. 3);
HSIPDNI (SEQ ID NO. 4);
HHMPHDK (SEQ ID NO. 5);
YTTPPSP (SEQ ID NO. 6); and
QLPLMPR (SEQ ID NO. 7),

wherein the peptide is capable of binding to Nogo (preferably Nogo-66), MAG and/or TN-R (preferably TNR-EGFL).

Preferably, the peptide is up to 50 amino acids in length, more preferably up to 40, up to 30, up to 25, up to 20, up to 19, up to 18, up to 17, up to 16, up to 15, up to 14, up to 13, up to 12, up to 11, up to 10, up to 9, or up to 8 amino acids in length.

A preferred peptide comprises SEQ ID NO. 1; another preferred peptide comprises SEQ ID NO. 3.

The invention further provides a peptide up to 60 amino acids in length comprising an amino acid sequence having at least 5 residues identical with corresponding residues in an amino acid sequence selected from the group consisting of:

YLTQPQS (SEQ ID NO. 1);
GSLPHSL (SEQ ID NO. 2);
TQLFPPQ (SEQ ID NO. 3);
HSIPDNI (SEQ ID NO. 4);
HHMPHDK (SEQ ID NO. 5);
YTTPPSP (SEQ ID NO. 6); and
QLPLMPR (SEQ ID NO. 7),

wherein the peptide is capable of binding to Nogo (preferably Nogo-66), MAG and/or TN-R (preferably TNR-EGFL).

Preferred sizes of the peptide are as stated previously. Peptides having at least 5 residues identical with corresponding residues of SEQ ID NO. 1, or at least 5 residues identical with corresponding residues of SEQ ID NO. 3 are preferred.

Preferably the minimum number of identical residues is 6.

The invention further provides a composition comprising one or more peptides of the invention, together with one or more pharmaceutically acceptable ingredients.

Preferably the composition is formulated for injection in vivo, preferably for direct injection into the CNS.

The invention further provides a peptide of the invention for use in a method of treatment. Such use may be in the treatment of CNS damage, especially spinal cord injury and stroke.

The invention also provides the use of a peptide of the invention in the preparation of a medicament for the treatment of CNS damage, especially spinal cord injury and stroke.

The invention also provides a method for treating CNS damage, the method comprising administering a peptide of the invention to a patient at or near a site of CNS damage in the patient. Specifically, the invention provides a method for treating SCI or stroke, the method comprising administering to a patient a peptide having an amino acid sequence that consists of SEQ ID NO. 1 or 3, by direct injection into a site of SCI or stroke damage in the patient.

The invention also provides the use of a peptide of the invention and/or a computer-generated model thereof, in the design of a mimetic capable of binding to one or more of the neuronal growth inhibitory molecules Nogo (preferably Nogo-66), MAG and/or TN-R (preferably TNR-EGFL).

Similarly, the invention provides a method of designing a mimetic of a peptide of the invention, the mimetic being capable of binding to one or more of the neuronal growth inhibitory molecules Nogo (preferably Nogo-66), MAG and/or TN-R (preferably TNR-EGFL), said method comprising:

(i) analysing a peptide of the invention that is capable of binding to one or more of the neuronal growth inhibitory molecules Nogo (preferably Nogo-66), MAG and/or TN-R (preferably TNR-EGFL) to determine the amino acid residues essential and important for the activity to define a pharmacophore; and

(ii) modelling the pharmacophore to design and/or screen candidate mimetics having the biological activity.

Preferably the method and/or use includes a step of assaying binding of a candidate mimetic to Nogo (preferably Nogo-66), MAG and/or TN-R (preferably TNR-EGFL) in vitro. Having identified a candidate mimetic that is capable of such in vitro binding, the candidate mimetic is preferably optimised for in vivo use. Following such optimisation, the optimised mimetic is preferably formulated together with one or more pharmaceutically acceptable ingredients.

The invention further provides a bacteriophage which expresses a fusion protein consisting of a peptide and a bacteriophage coat protein, such that the peptide is displayed on the surface of the bacteriophage virion, wherein the peptide is up to 60 amino acids in length and comprises an amino acid sequence having at least four residues identical with corresponding residues of an amino acid sequence selected from the group consisting of:

YLTQPQS (SEQ ID NO. 1);
GSLPHSL (SEQ ID NO. 2);
TQLFPPQ (SEQ ID NO. 3);
HSIPDNI (SEQ ID NO. 4);
HHMPHDK (SEQ ID NO. 5);
YTTPPSP (SEQ ID NO. 6); and
QLPLMPR (SEQ ID NO. 7).

Preferably the peptide of the invention is up to 50 amino acids in length, more preferably up to 40, up to 30, up to 25, up to 20, or up to 15 amino acids in length. Still more preferably, the peptide of the invention is 8-12 amino acids in length, more preferably 6-10. Preferably the minimum number of identical residues is 5 or 6.

The invention further provides a screening method for peptides capable of binding to Nogo (preferably Nogo-66), MAG and/or TN-R (preferably TNR-EGFL), the method comprising:

providing bacteriophages of the invention, respectively expressing different peptides; and

screening the bacteriophages for the ability to bind to Nogo (preferably Nogo-66), MAG and/or TN-R (preferably TNR-EGFL).

Bacteriophages which are identified as being capable of binding to Nogo (preferably Nogo-66), MAG and/or TN-R (preferably TNR-EGFL), or the peptides they display, may then be screened for the ability to block the inhibitory effects of Nogo (preferably Nogo-66), MAG and/or TN-R (preferably TNR-EGFL) on neuronal cell adhesion in an in vitro assay. Following the identification of a peptide (or phage that displays a peptide) that is capable of blocking the inhibitory effects of Nogo (preferably Nogo-66), MAG and/or TN-R (preferably TNR-EGFL) on neuronal cell adhesion in an in vitro assay, the peptide is preferably formulated with one or more pharmaceutically acceptable ingredients for administration in vivo.

The invention further provides a method of searching for factors that are likely to reduce the inhibitory effect of TN-R, MAG and/or Nogo, the method comprising interrogating a sequence database to identify polypeptides, or nucleic acids that encode polypeptides, that comprise an amino acid sequence having at least 5 residues identical with corresponding residues in an amino acid sequence selected from the group consisting of:

YLTQPQS (SEQ ID NO. 1);

GSLPHSL (SEQ ID NO. 2);
TQLFPPQ (SEQ ID NO. 3);
HSIPDNI (SEQ ID NO. 4);
HHMPHDK (SEQ ID NO. 5);
YTTPPSP (SEQ ID NO. 6); and
QLPLMPR (SEQ ID NO. 7).

The database is preferably a cDNA database. It may be an EST database. Preferably it is a database of sequences expressed in mammalian CNS. Less specific databases may be used, although this may generate more false positive results.

The invention further provides a method of searching for factors that are likely to reduce the inhibitory effect of TN-R, MAG and/or Nogo, the method comprising screening a cDNA library with an oligonucleotide probe which is capable of hybridising under stringent conditions with a nucleic acid sequence that encodes an amino acid sequence having at least 5 residues identical with corresponding residues in an amino acid sequence selected from the group consisting of:

YLTQPQS (SEQ ID NO. 1);
GSLPHSL (SEQ ID NO. 2);
TQLFPPQ (SEQ ID NO. 3);
HSIPDNI (SEQ ID NO. 4);
HHMPHDK (SEQ ID NO. 5);
YTTPPSP (SEQ ID NO. 6); and
QLPLMPR (SEQ ID NO. 7).

The cDNA library is preferably mammalian, more preferably human. The library is preferably derived from CNS tissue.

Each of the two preceding methods preferably further includes a step, following the identification of a candidate

polypeptide, or nucleic acid encoding the candidate polypeptide, of testing the polypeptide for the ability to reduce the inhibitory effect of TN-R, MAG and/or Nogo. Preferred further steps are as indicated above.

Second aspect

In this aspect, the invention provides nucleic acid vector comprising nucleic acid encoding one or more polypeptide domains selected from the group consisting of:

(a) the N-terminal (NogoN) domain of Nogo-A;

(b) the extracellular loop (Nogo66) of Nogo-B (also present in Nogo-A and Nogo-C);

(c) the third to fifth immunoglobulin-like repeats of MAG; and

(d) the EGF-like domain of TN-R.

The TN-R EGF-L domain has been identified in Xiao et al. (1996) as being capable of inhibiting neurite outgrowth in vitro of a neuronal cell line and primary neurons.

The vector will include control sequences necessary for the expression of said nucleic acid in mammalian cells. Preferably the vector is a commercially available vaccine vector, into which said nucleic acid has been inserted. Suitable and preferred commercially available vaccine vectors include the pcDNA 3.1 family of vectors, especially pcDNA 3.1$^+$, and pVAX1 (all from Invitrogen, San Diego, California, US).

Usually the nucleic acid will be DNA.

Preferably the nucleic acid encodes at least two of the domains, more preferably at least three, more preferably it

encodes all four. The nucleic acid may encode a plurality of copies of any one or more of the domains.

Where the nucleic acid encodes more than one domain, the domains are preferably expressed as a fusion polypeptide. Preferably the domains are separated from one another by flexible linkers (preferably poly-Ala linkers, e.g. Ala_3 linkers) to facilitate correct folding of the domains.

Of the proteins Nogo A, Nogo B, TN-R and/or MAG (and/or preferably Nogo-C), the vector is preferably capable of expressing substantially only the domains indicated. NogoN is a domain of the Nogo A isoform. Nogo 66 is a domain found in all three isoforms, Nogo-A, Nogo-B and Nogo-C. In particular, the vector is preferably incapable of expressing other epitope-containing portions of the proteins. Of the proteins Nogo A, Nogo B, TN-R and/or MAG (and/or preferably Nogo-C), the vector preferably expresses no more than 20%, more preferably no more than 15%, no more than 12%, no more than 10%, no more than 8%, no more than 6%, no more than 5%, no more than 4%, no more than 3%, no more than 2% or no more than 1% of the protein lying outside the domains listed above. This is particularly preferred for vectors containing a domain or domains of only one of the proteins.

The amino acid sequences of Nogo A, Nogo B, Nogo C, TN-R and MAG are available from GenBank, for example under the following accession numbers:

MAG - X05301 (GI 56611)
TN-R - Z67996 (GI 1261914)
Nogo B - AJ251384 (GI 9408097)
Nogo A - AF320999 (GI 11878297)
Nogo C - CAB99250 (GI 9408100)

The domains preferably have the amino acid sequences shown below, i.e. for domain (c), the 508 residue amino acid sequence of MAG (1-508); for domain (d), the 205 residue amino acid sequence of TNR (125-329); for domain (a), the 185 amino acid sequence of NogoN (1-185); and for domain (b), the 66 amino acid sequence of Nogo66 (823-888).

MAG (1-508)

MIFLTTLPLF WIMISASRGG HWGAWMPSSI SAFEGTCVSI PCRFDFPDEL
RPAVVHGVWY FNSPYPKNYP PVVFKSRTQV VHESFQGRSR LLGDLGLRNC
TLLLSTLSPE LGGKYYFRGD LGGYNQYTFS EHSVLDIINT PNIVVPPEVV
AGTEVEVSCM VPDNCPELRP ELSWLGHEGL GEPTVLGRLR EDEGTWVQVS
LLHFVPTREA NGHRLGCQAA FPNTTLQFEG YASLDVKYPP VIVEMNSSVE
AIEGSHVSLL CGADSNPPPL LTWMRDGMVL REAVAESLYL DLEEVTPAED
GIYACLAENA YGQDNRTVEL SVMYAPWKPT VNGTVVAVEG ETVSILCSTQ
SNPDPILTIF KEKQILATVI YESQLQLELP AVTPEDDGEY WCVAENQYGQ
RATAFNLSVE FAPIILLESH CAAARDTVQC LCVVKSNPEP SVAFELPSRN
VTVNETEREF VYSERSGLLL TSILTLRGQA QAPPRVICTS RNLYGTQSLE
LPFQGAHR (SEQ ID NO:8)

TNR (125-329)

CPCASS AQVLQELLSR IEMLEREVSV LRDQCNANCC QESAATGQLD
YIPHCSGHGN FSFESCGCIC NEGWFGKNCS EPYCPLGCSS RGVCVDGQCI
CDSEYSGDDC SELRCPTDCS SRGLCVDGEC VCEEPYTGED CRELRCPGDC
SGKGRCANGT CLCEEGYVGE DCGQRQCLNA CSGRGQCEEG LCVCEEGYQG
PDCSAVAPP (SEQ ID NO:9)

NogoN (1-185)

MEDLDQSPLV SSSDSPPRPQ PAFKYQFVRE PEDEEEEEEE EEEDEDEDLE
ELEVLERKPA AGLSAAPVPT APAAGAPLMD FGNDFVPPAP RGPLPAAPPV
APERQPSWDP SPVSSTVPAP SPLSAAAVSP SKLPEDDEPP ARPPPPPPAS
VSPQAEPVWT PPAPAPAAPP STPAAPKRRG SSGSV (SEQ ID NO:10)

Nogo66 (823-888)

RIYKGVIQ AIQKSDEGHP FRAYLESEVA ISEELVQKYS NSALGHVNCT
IKELRRLFLV DDLVDSLK (SEQ ID NO:11)

The vector preferably encodes a polypeptide having the amino acid sequence MAG(1-508)-Ala_n-TNR(125-329)-Ala_n-NogoN(1-

185)-Ala_n-Nogo66(823-888) (SEQ ID NO:12), where Ala_n represents an polyalanine linker. n is preferably 3.

The domains are preferably encoded by the following sequences, i.e. for domain (c), the 508 codon nucleic acid sequence of MAG (126-1649); for domain (d), the 205 codon nucleic acid sequence of TNR (454-1068); for domain (a), the 185 codon nucleic acid sequence of NogoN (1-555); and/or for domain (b), the 66 codon nucleic acid sequence of Nogo66 (2467-2664):

MAG (126-1649)

atgat attccttacc accctgcctc tgttttggat aatgatttca
gcttctcgag gggggcactg gggtgcctgg atgccctcgt ccatctcagc
cttcgagggc acgtgtgtct ccatcccctg ccgtttcgac ttcccggatg
agctcagacc ggctgtggta catggcgtct ggtatttcaa cagtccctac
cccaagaact acccgccagt ggtcttcaag tcccgcacac aagtggtcca
cgagagcttc cagggccgta gccgcctgtt gggagacctg ggcctacgaa
actgcaccct gcttctcagc acgctgagcc ctgagctggg agggaaatac
tatttccgag gtgacctggg cggctacaac cagtacacct tctcggagca
cagcgtcctg gacatcatca acacccccaa catcgtggtg cccccagaag
tggtggcagg aacggaagta gaggtcagct gcatggtgcc ggacaactgc
ccagagctgc gccctgagct gagctggctg ggccacgagg ggctagggga
gcccactgtt ctgggtcggc tgcgggagga tgaaggcacc tgggtgcagg
tgtcactgct acacttcgtg cctactagag aggccaacgg ccaccgtctg
ggctgtcagg ctgccttccc caacaccacc ttgcagttcg agggttacgc
cagtctggac gtcaagtacc ccccggtgat tgtggagatg aattcctctg
tggaggccat tgagggctcc cacgtcagcc tgctctgtgg ggctgacagc
aacccgccac cgctgctgac ttggatgcgg gatgggatgg tgttgaggga
ggcagttgct gagagcctgt acctggatct ggaggaggtg accccagcag
aggacggcat ctatgcttgc ctggcagaga atgcctatgg ccaggacaac
cgcacggtgg agctgagcgt catgtatgca ccttggaagc ccacagtgaa
tgggacggtg gtggcggtag agggggagac agtctccatc ctgtgttcca
cacagagcaa cccggaccct attctcacca tcttcaagga gaagcagatc
ctggccacgg tcatctatga gagtcagctg cagctggaac tccctgcagt
gacgcccgag gacgatgggg agtactggtg tgtagctgag aaccagtatg
gccagagagc caccgccttc aacctgtctg tggagtttgc tcccataatc
cttctggaat cgcactgtgc agcggccaga gacaccgtgc agtgcctgtg
tgtggtaaaa tccaacccgg aaccctccgt ggcctttgag ctgccttccc
gcaacgtgac tgtgaacgag acagagaggg agtttgtgta ctcagagcgc
agcggcctcc tgctcaccag catcctcacg ctccggggtc aggcccaagc
cccaccccgc gtcatttgta cctccaggaa cctctacggc acccagagcc
tcgagctgcc tttccaggga gcacaccga (SEQ ID NO:13)

This sequence commences with a start codon; if any other sequence is used at the 5′ end of the nucleic acid, a start codon will be required. It is of course a matter of routine to engineer this into any nucleic acid sequence of interest, though it will be noted that Nogo(1-555) also commences with a start codon.

TNR (454-1068)

tgtccat gtgccagttc agcccaggtg ctgcaggagc tgctgagccg gatcgagatg ctggagaggg aggtgtcggt gctgcgagac cagtgcaacg ccaactgctg ccaagaaagt gctgccacag gacaactgga ctatatccct cactgcagtg gccacggcaa ctttagcttt gagtcctgtg gctgcatctg caacgaaggc tggtttggca agaattgctc ggagccctac tgcccgctgg gttgctccag ccgggggggtg tgtgtggatg gccagtgcat ctgtgacagc gaatacagcg gggatgactg ttccgaactc cggtgcccaa cagactgcag ctcccggggg ctctgcgtgg acggggagtg tgtctgtgaa gagccctaca ctggcgagga ctgcagggaa ctgaggtgcc ctggggactg ttcggggaag gggagatgtg ccaacggtac ctgtttatgc gaggagggct acgttggtga ggactgcggc cagcggcagt gtctgaatgc ctgcagtggg cgaggacaat gtgaggaggg gctctgcgtc tgtgaagagg gctaccaggg ccctgactgc tcagcagttg cccctcca (SEQ ID NO:14)

NogoN (1-555)

atggaagacc tggaccagtc tcctctggtc tcgtcctcgg acagcccacc ccggccgcag cccgcgttca agtaccagtt cgtgagggag cccgaggacg aggaggaaga agaggaggag gaagaggagg acgaggacga agacctggag gagctggagg tgctggagag gaagcccgcc gccgggctgt ccgcggcccc agtgcccacc gcccctgccg ccggcgcgcc cctgatggac ttcggaaatg acttcgtgcc gccggcgccc cggggacccc tgccggccgc tccccccgtc gccccggagc ggcagccgtc ttgggacccg agcccggtgt cgtcgaccgt gcccgcgcca tccccgctgt ctgctgccgc agtctcgccc tccaagctcc ctgaggacga cgagcctccg gcccggcctc cccctcctcc cccggccagc gtgagccccc aggcagagcc cgtgtggacc ccgccagccc cggctcccgc cgcgcccccc tccaccccgg ccgcgcccaa gcgcaggggc tcctcgggct cagtg (SEQ ID NO:15)

Nogo66 (2467-2664)

agga tatacaaggg tgtgatccaa gctatccaga aatcagatga aggccaccca ttcagggcat atctggaatc tgaagttgct atatctgagg agttggttca gaagtacagt aattctgctc ttggtcatgt gaactgcacg ataaaggaac tcaggcgcct cttcttagtt gatgatttag ttgattctct gaag (SEQ ID NO:16)

It is, however, thought that fragments, derivatives or variants of these specific domains will also give rise to effective vaccines. Accordingly, a polypeptide domain within the meaning of the invention may be a fragment of any one of the four amino acid sequences given above, the fragment preferably consisting of at least 15 contiguous amino acids from said sequence, more preferably at least 17, more preferably at least 20, 25, 30, 40, 50 or 60 amino acids. For MAG, NogoN and TNR, the size of the fragment is more preferably at least 80 amino acids, more preferably 100, 120, 140, 160 or 180 amino acids. For TNR and MAG, the length is more preferably 200 amino acids. For MAG, the length is more preferably 250, 300, 350, 400 or 450 amino acids. The fragment will include one or more epitopes of the specific domain whose sequence is given above, and will retain the ability of the domain to raise an antibody response in vivo. Thus, the fragment will be capable of raising antibodies in vivo that cross-react with the corresponding domain. Linear epitope mapping is a matter of routine for the skilled person.

Similarly, a polypeptide domain within the meaning of the invention may be a variant of any one amino acid sequence (the reference amino acid sequence) given above. In this context, a variant is a polypeptide having a sequence which differs from the reference amino acid sequence, but which includes a portion of at least 15 amino acids that has at least 65% amino acid identity to a corresponding portion of the reference sequence. Preferably the level of identity is at least 70%, 75%, 80%, 85%, 90%, 95%, 98% or 99%. Preferably the portion is at least 17 amino acids in length, more preferred lengths are those given in the preceding paragraph for the lengths of fragments. Again, the variant will generally include one or more epitopes of the specific

domain whose sequence is given above, and will retain the ability of the domain to raise an antibody response in vivo. Thus, the variant will be capable of raising antibodies in vivo that cross-react with the corresponding domain.

The invention also provides a composition comprising the vector of the invention, formulated together with one or more pharmaceutically acceptable ingredients for use as a (therapeutic) vaccine. Preferably the composition is formulated for administration by injection.

The invention further provides the vector of the invention, for use in a method of treatment. The treatment may be of CNS damage, especially SCI or stroke.

The invention also provides the use of the vector of the invention in the manufacture of a medicament for the treatment of CNS damage, especially SCI and stroke.

The invention also provides a method for treating CNS damage in a patient, the method comprising administering a vector of the invention to the patient as a therapeutic vaccine.

The vectors of the invention will typically be administered by injection, although other vaccine delivery methods are known in the art (such as oral or transdermal delivery and "needle-free" injection) and may be used in accordance with the invention. Injection will typically be intra-muscular. While the blood-brain barrier normally provides an obstacle to the passage of antibodies, CNS damage (especially injury, e.g. external injury) will generally allow passage of antibodies across the blood-brain barrier at the site of damage. It is considered therefore that no special measures are required to allow passage across the blood-brain barrier

of antibodies raised in response to vaccination with the vector of the invention.

The invention further provides a polypeptide consisting essentially of one or more polypeptide domains selected from the group consisting of:

(a) the N-terminal (NogoN) domain of Nogo-A;

(b) the extracellular loop of Nogo-B (also present in Nogo-A and Nogo-C);

(c) the third to fifth immunoglobulin-like repeats of MAG; and

(d) the EGF-like domain of TN-R.

Preferred features of the polypeptide are as defined above for the polypeptide encoded by the vector.

The invention also provides a composition comprising the polypeptide of the invention, formulated together with one or more pharmaceutically acceptable ingredients for use as a (therapeutic) vaccine. Preferably the composition is formulated for administration by injection.

The invention further provides the polypeptide of the invention, for use in a method of treatment. The treatment may be of CNS damage, especially SCI or stroke.

The invention also provides the use of the polypeptide of the invention in the manufacture of a medicament for the treatment of CNS damage, especially SCI or stroke.

The invention also provides a method for treating CNS damage in a patient, the method comprising administering a polypeptide of the invention to the patient as a therapeutic vaccine.

Administration is preferably as described for the vector.

The invention further provides analogous methods and uses of antibodies capable of specifically binding to any one domains (a)-(d), or preferably mixtures of antibodies together capable of binding two, three or all four of domains (a)-(d) for the treatment of CNS damage, especially SCI or stroke. The antibodies will be administered directly to the patient (preferably as for the peptides of the first aspect, e.g. by direct injection into the CNS, into the site of damage or into the cerebro-spinal fluid), instead of administering a nucleic acid or polypeptide vaccine to generate an antibody response. Antibody fragments capable of specific binding are regarded to be antibodies for this purpose.

Detailed description

Peptides

The term "peptide" is intended to refer to a molecule consisting of several amino acids, adjacent pairs of amino acids being linked by peptide bonds. A peptide bond has the structure -CO-NH-. Amino acids may be naturally occurring or non-naturally occurring. Terminal amino acids may include terminal modifications. Naturally occurring chiral amino acids (i.e. amino acids other than non-chiral glycine) are of the L-isoform. Peptides of the invention, however, may include or consist of amino acids of the D-isoform. Such D-amino acids may be the D-isoforms of naturally occurring L-amino acids, or may have no naturally occurring L-isoform. The inclusion of D-amino acids in the peptides

of the invention may assist in reducing clearance of the peptide in vivo.

Synthesis of peptides

Peptides may be generated wholly or partly by chemical synthesis. The peptides of the invention can be readily prepared according to well-established, standard liquid or, preferably, solid-phase peptide synthesis methods, general descriptions of which are broadly available (see, for example, in J.M. Stewart and J.D. Young, Solid Phase Peptide Synthesis, 2nd edition, Pierce Chemical Company, Rockford, Illinois (1984), in M. Bodanzsky and A. Bodanzsky, The Practice of Peptide Synthesis, Springer Verlag, New York (1984); and Applied Biosystems 430A Users Manual, ABI Inc., Foster City, California), or they may be prepared in solution, by the liquid phase method or by any combination of solid-phase, liquid phase and solution chemistry, e.g. by first completing the respective peptide portion and then, if desired and appropriate, after removal of any protecting groups being present, by introduction of the residue X by reaction of the respective carbonic or sulfonic acid or a reactive derivative thereof.

Phage Display

Phage display is a recent and promising technique in biomolecular engineering which can survey tens of millions of short peptides for tight binding to an antibody receptor or other binding protein. Phage display was firstly described in 1985 (Smith et al, 1985) and is a selection technique in which a peptide or protein is expressed as a fusion with a coat protein of a bacteriophage, resulting in display of the fused protein on the surface of the virion,

while the DNA encoding the fusion resides within the virion. Phage display creates a physical linkage between a vast library of random peptide sequences and DNA encoding each sequence, allowing rapid identification of peptide ligands for a variety of target molecules by an in vitro selection process called panning (Scott et al, 1990; Arap et al 1998).

Interactions that involve particular protein domains (either protein/protein or protein/non-protein interactions) typically only need 8-12 amino acids, among which 5-8 amino acids play a key role. Phage display libraries displaying peptides of 6-10 amino acid residues have been successfully used in a number of applications, including epitope mapping, mapping protein-protein contacts, identification of peptide mimetics of non-peptide ligands and design of novel vaccine and new drugs (Scott et al, 1990; Cwirla et al, 1990; Devlin et al 1990 Felici et al, 1991; Motti et al, 1994; Hong et al, 1995; Arap et al, 1998; Nilsson et al, 2000).

Recent work has shown that a series of peptides isolated using a broad range of enzymes as targets contained similar amino acid sequences for each target and bound one or two sites per target by competition analysis. Of 17 peptides tested, 13 were found to be specific inhibitors of enzyme function. Peptidic surrogate ligands identified using phage display are preferentially targeted to a limited number of sites that inhibit enzyme function (Hyde-DeRuyscher et al 2000).

Mimetics

Non-peptide "small molecules" are often preferred to peptides for in vivo pharmaceutical use. Accordingly,

mimetics of the peptides of the invention may be designed for pharmaceutical use.

The designing of mimetics to a known pharmaceutically active compound is a known approach to the development of pharmaceuticals based on a "lead" compound. This might be desirable where the active compound is difficult or expensive to synthesise or where it is unsuitable for a particular method of administration, e.g. peptides are unsuitable active agents for oral compositions as they tend to be quickly degraded by proteases in the alimentary canal. Mimetic design, synthesis and testing is generally used to avoid randomly screening large number of molecules for a target property.

There are several steps commonly taken in the design of a mimetic from a compound having a given target property. Firstly, the particular parts of the compound that are critical and/or important in determining the target property are determined. In the case of a peptide, this can be done by systematically varying the amino acid residues in the peptide, e.g. by substituting each residue in turn. Alanine scans of peptide are commonly used to refine such peptide motifs. These parts or residues constituting the active region of the compound are known as its "pharmacophore".

Once the pharmacophore has been found, its structure is modelled according to its physical properties, eg stereochemistry, bonding, size and/or charge, using data from a range of sources, eg spectroscopic techniques, X-ray diffraction data and NMR. Computational analysis, similarity mapping (which models the charge and/or volume of a pharmacophore, rather than the bonding between atoms) and other techniques can be used in this modelling process.

In a variant of this approach, the three-dimensional structure of the ligand and its binding partner are modelled. This can be especially useful where the ligand and/or binding partner change conformation on binding, allowing the model to take account of this in the design of the mimetic.

A template molecule is then selected onto which chemical groups which mimic the pharmacophore can be grafted. The template molecule and the chemical groups grafted on to it can conveniently be selected so that the mimetic is easy to synthesise, is likely to be pharmacologically acceptable, and does not degrade *in vivo*, while retaining the biological activity of the lead compound. Alternatively, where the mimetic is peptide based, further stability can be achieved by cyclising the peptide, increasing its rigidity. The mimetic or mimetics found by this approach can then be screened to see whether they have the target property, or to what extent they exhibit it. Further optimisation or modification can then be carried out to arrive at one or more final mimetics for in vivo or clinical testing.

Sequence identity

Percent (%) amino acid sequence identity with respect to a reference sequence is defined as the percentage of amino acid residues in a candidate sequence that are identical with the amino acid residues in the reference sequence, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity, and not considering any conservative substitutions as part of the sequence identity. % identity values may be determined by WU-BLAST-2 (Altschul et al., Methods in

Enzymology, 266:460-480 (1996)). WU-BLAST-2 uses several search parameters, most of which are set to the default values. The adjustable parameters are set with the following values: overlap span = 1, overlap fraction = 0.125, word threshold (T) = 11. A % amino acid sequence identity value is determined by the number of matching identical residues as determined by WU-BLAST-2, divided by the total number of residues of the reference sequence (gaps introduced by WU-BLAST-2 into the reference sequence to maximize the alignment score being ignored), multiplied by 100.

Stringent conditions

Stringent conditions may be identified by those that: (1) employ low ionic strength and high temperature for washing, for example 0.015 M sodium chloride/0.0015 M sodium citrate/ 0.1% sodium dodecyl sulfate at 50°C; (2) employ during hybridization a denaturing agent, such as formamide, for example 50% (v/v) formamide with 0.1% bovine serum albumin/0.1% Ficoll/0.1% polyvinylpyrrolidone/50mM sodium phosphate buffer at pH 6.5 with 760 mM sodium chloride, 75 mM sodium citrate at 42°C; or (3) employ 50% formamide, 5 x SSC (0.75 M NaCl, 0.075 M sodium citrate), 50 mM sodium phosphate (pH 6.8), 0.1% sodium pyrophosphate, 5 x Denhardt's solution, sonicated salmon sperm DNA (50 µg/ml), 0.1% SDS, and 10% dextran sulfate at 42°C, with washes at 42°C in 0.2 x SSC (sodium chloride/sodium citrate) and 50% formamide at 55°C, followed by a high-stringency wash consisting of 0.1 x SSC containing EDTA at 55°C.

The subject

The subject to which the compositions and/or treatments of the invention will be administered will be a mammal,

preferably an experimental animal such as a rodent (e.g. a rabbit, rat or mouse), dog, cat, monkey or ape, or a farm animal such as a cow, horse, sheep, pig or goat. More preferably, the subject is human.

Generally, the subject will have CNS damage, usually CNS injury, e.g. a head injury. More preferably, however, the damage is to the spinal cord, e.g. SCI. In experimental animals, the damage may be experimental. The CNS damage may also result from a disease or disorder, e.g. epilepsy, stroke, or a neurodegenerative condition, learning memory-related condition and/or dementia such as Alzheimer's disease or Parkinson's disease.

The treatments of the invention will generally be intended for use in conjunction with other therapies, such as surgery and/or rehabilitation.

Formulations

It is preferable to present the peptides, mimetics, and vectors of the invention as pharmaceutical formulations (e.g., composition, preparation, medicament) comprising at least one active compound, as defined above, together with one or more other pharmaceutically acceptable ingredients well known to those skilled in the art, including, but not limited to, pharmaceutically acceptable carriers, adjuvants, excipients, buffers, preservatives and stabilisers. The formulation may further comprise other active agents.

Thus, the present invention further provides a method of making a pharmaceutical composition as previously defined, the method comprising admixing at least one peptide or vector of the invention together with one or more

pharmaceutically acceptable ingredients well known to those skilled in the art, e.g., carriers, adjuvants, excipients, etc..

The term "pharmaceutically acceptable" as used herein pertains to compounds, ingredients, materials, compositions, dosage forms, etc., which are, within the scope of sound medical judgment, suitable for use in contact with the tissues of the subject in question (e.g., human) without excessive toxicity, irritation, allergic response, or other problem or complication, commensurate with a reasonable benefit/risk ratio. Each carrier, adjuvant, excipient, etc. must also be "acceptable" in the sense of being compatible with the other ingredients of the formulation.

Suitable carriers, adjuvants, excipients, etc. can be found in standard pharmaceutical texts, for example Remington's Pharmaceutical Sciences, 20th Edition, 2000, pub. Lippincott, Williams & Wilkins; and Handbook of Pharmaceutical Excipients, 2nd edition, 1994.

Formulations may suitably be injectable formulations, e.g. in the form of aqueous, isotonic, pyrogen-free, sterile solutions, in which the active compound is dissolved. Such liquids may additional contain other pharmaceutically acceptable ingredients, such as anti-oxidants, buffers, preservatives, stabilisers, bacteriostats, suspending agents, thickening agents, and solutes which render the formulation isotonic with the blood or cerebrospinal fluid. Examples of suitable isotonic carriers for use in such formulations include Sodium Chloride Injection, Ringer's Solution, or Lactated Ringer's Injection. Typically, the concentration of the active compound in the liquid is from about 1 ng/ml to about 10 µg/ml, for example from about

10 ng/ml to about 1 µg/ml. The formulations may be presented in unit-dose or multi-dose sealed containers, for example, ampoules and vials, and may be stored in a freeze-dried (lyophilised) condition requiring only the addition of the sterile liquid carrier, for example water for injections, immediately prior to use. Extemporaneous injection solutions and suspensions may be prepared from sterile powders, granules, and tablets.

Administration

Administration of the peptides of the invention will generally be by injection, preferably directly into the CNS. Injection may be directly into the site of damage, e.g. into the site of damage. Alternatively, injection may be into the cerebro-spinal fluid, typically near the site of injury.

Experimental work underlying the invention and embodiments of the invention will now be described, by way of example only, with reference to the accompanying **Figure 1**, which shows the primers used to isolate the inhibitory domains from the proteins MAG, TN-R and Nogo. Coding sequence (or the complementary strand) is shown in bold. Start (Met) and stop (***) codons are doubly underlined. Restriction sites are underlined. SEQ ID NOS are as follows:

Primer	SEQ ID NO
MAG1	17
MAG2	18
TNR1	19
TNR2	20
NogoN1	21
NogoN2	22
Nogo66-1	23

Nogo66-2 24

Also shown schematically is the resultant construct (SEQ ID NO:25) obtained by digestion and sequential connection of the resultant PCR products. Restriction sites are underlined. Ala-encoding codons are labelled. Coding sequence for each inhibitory domain is represented by the bracketed name of the domain. The stop codon is labelled ***.

Aspect 1, Example 1

Identification of Nogo domains responsible having inhibitory properties

Repulsion assay

NG108 cells (mouse neuroblastoma - rat glioma hybrid cells, commercially available from the American Tissue Type Culture Collection, Manassas, VA, USA, under accession number ATCC HB-12317) were plated on coated substrates using Nogo1-25, Nogo1-50, Nogo1-66 and GST-Nogo-66 as well as GST as control. Generation and purification of the recombinant domains of Nogo, either alone or as fusion proteins with GST, were performed as previously described (Xiao ZC et al, 1996).

NG108 cells were repelled significantly from Nogo1-50, Nogo1-66, and GST-Nogo-66, but not Nogo1-25 and GST.

Neurite outgrowth assay

NG108 cells were plated on coated substrates using Nogo1-25, Nogo1-50, Nogo1-66, and GST-Nogo-66 as well as GST as control.

Neurite outgrowth of NG108 cells were inhibited significantly from Nogo1-50, Nogo1-66, and GST Nogo-66, but not Nogo1-25 and GST.

Aspect 1, Example 2

Screening of novel short peptides against neuronal growth inhibitory molecules TN-R, MAG, and Nogo through phage display technology

Purification of TN-R, MAG and Nogo from adult mouse brains was performed by immumoaffinity chromatography as previously described (Pesheva, P et al, 1989). Generation and purification of the recombinant domains of TN-R, MAG, Nogo as fusion proteins with GST were performed as previously described (Xiao ZC et al, 1996). All these proteins were used as phage binding targets for rapid screening, using the Ph.D-7TM phage display peptide library kit (New England Biolabs, Ltd) according to the manufacturer's instructions.

Briefly, a library of phages, displaying different peptide sequences, was exposed to a plate coated with the target protein. Unbound phage was washed away and specifically bound phage was eluted by lowering pH. The eluted pool of phage was amplified, and the process was repeated 3-4 times.

After 3-4 rounds of affinity selection, several specific phage clones were isolated from 7-mer random peptide phage-displayed libraries and identified by ELISA. The peptide-

encoding sequences of the specific phage clones were determined by automated sequencing. The sequences of peptides which bound specifically to the targeted proteins were obtained.

The framework sequence of the phage coat protein, into which a 7-mer peptide-encoding sequence is inserted in each phage, is as follows:

TTA TTC GCA ATT CCT TTA GTG GTA CCT TTC TAT TCT CAC TCT (SEQ ID NO:26) … GGT GGA GGT TCG GCC GAA ACT GTT GAA AGT TGT (SEQ ID NO:27)

… represents the site of insertion of the 7-mer peptide-encoding sequence. A Kpn1 site is underlined, a Eag1 site is doubly underlined.

Exemplary 7-mer peptide-encoding sequences are shown below. "n" refers to the number of isolated phages displaying a peptide having the sequence shown (though there was of course some variability in the encoding nucleic acid sequences, owing to the degeneracy of the genetic code). "SIN" is an abbreviation for "SEQ ID NO." and refers to the peptide sequence. The SEQ ID NOS of the nucleic acid sequences are 28-35.

Target	Exemplary nucleic acid	Peptide	SIN
Nogo-66	TAT CTG ACG CAG CCT CAG TCG	YLTQPQS (n=43)	1
Nogo-66	GGT TCT CTG CCT CAT TCG CTG	GSLPHSL (n=8)	2
TNR-EGFL	ACG CAG CTG TTT CCT CCT TAG	TQLFPPQ (n=18)	3
TNR-EGFL	CAT TCT ATT CCT GAT AAT ATT	HSIPDNI (n=3)	4
TNR-EGFL	CAT CAT ATG CCT CAT GAT AAG	HHMPHDK (n=1)	5
TNR-EGFL	GGT TCT CTG CCT CAT TCG CTG	GSLPHSL (n=1)	2
TNR-EGFL	TAT ACG ACG CCT CCG AGT CCT	YTTPPSP (n=1)	6

MAG	TAT CTG ACG CAG CCT CAG TCG	YLTQPQS (n=19)	1
MAG	CAG CTT CCG CTT ATG CCT CGT	QLPLMPR (n=5)	7
MAG	ACG CAG CTG TTT CCT CCT CAG	TQLFPPQ (n=7)	3

5

Aspect 1, Example 3

In vitro assay for phages displaying peptides that reduce the inhibitory effect of Nogo, MAG and TN-R on neuronal adhesion

Tissue culture petri dishes (Becton Dickinson) with a diameter of 3.5 cm were coated with methanol-solubilized nitrocellulose according to Lagenaur and Lemmon (1987) and air-dried under a sterile hood. Then the petri dishes were incubated with PBS containing 5 µg/ml poly-DL-ornithine for 2 hours at 37°C. Subsequently, the dishes were washed three times with PBS and dried under a sterile hood.

Protein spots (1.5 µl of 5 µM GST, 5 µM GST-Nogo66, 100µM Nogo66, 100 µM Nogo1-50 or 100 µM Nogo1-25) were applied to the nitrocellulose-coated surfaces of the petri dishes and incubated for 2 hours at 37°C in a humidified atmosphere. Subsequently, the spots were washed three times with PBS. The dishes were then flooded with PBS containing 2% heat-inactivated fatty acid-free BSA (Sigma) and incubated overnight to block residual non-specific protein binding sites.

Then, the dishes were washed with PBS and NG108 cells were plated in 2 ml of Dulbecco's modified Eagle's medium containing 10% fetal bovine serum at a density of 2.5×10^5 cells/ml and incubated at 37°C in a humidified atmosphere. After 12 hours, the dishes were gently washed three times with PBS and the cells fixed by flooding with PBS containing 2.5% glutaraldehyde. After fixation, cultures were stained with 0.5% toluidine blue (Sigma) in 2.5% sodium carbonate. The stained cultures were then washed three times with water and air-dried.

Cells adhering to the various spots were photographed and counted. All experiments were performed at least three times. Statistical analysis was carried out by Student test. The level of significance was chosen as $p < 0.05$.

GST-Nogo66, Nogo66 and Nogo1-50 all displayed significantly reduced NG108 cell counts compared to GST; Nogo1-25 did not.

NG108 cells were plated on poly-DL-ornithine-treated tissue culture petri dish as single-cell suspensions. Specific phages that display the Nogo66-, MAG- and TNR-binding peptides identified above were added to the cell culture. Cells were maintained for 24 hours before fixation and staining with toluidine blue. Cell adhesion was determined as previously described.

Phage displaying the peptide YLTQPQS (SEQ ID NO. 1) was able to block the inhibitory effects of Nogo-66 and MAG on NG108 cell binding; phage displaying the peptide TQLFPPQ (SEQ ID NO. 3) was able to block the inhibitory effect of TNR-EGFL on NG108 cell binding.

Aspect 1, Example 4

In vitro assay for peptides that reduce the inhibitory effect of Nogo, MAG and TN-R on neuronal adhesion

All the screened consensus peptides are synthesized using the solid-phase method by means of a Peptide Synthesizer Model 1000. Neurite outgrowth and growth cone repulsion assays are performed as described previously (Xiao ZC et al, 1997). Purified intact TN-R, MAG and Nogo and recombinant

domains of TN-R, MAG and Nogo are be coated as substrate, to allow identification of the inhibitory domains of TN-R, MAG and Nogo. In a further assay, the synthesized peptides are mixed with the relevant protein/domain before coating, to identify peptides that can neutralize the inhibitory effects of TN-R, MAG and/or Nogo in vitro.

Aspect 1, Example 5

In vivo test for peptides that can alleviate spinal cord injury

Those consensus short peptides that can neutralize the inhibitory effects of TN-R, MAG and/or Nogo in vitro are used in vivo to determine that they alleviate spinal cord injury (Bregman et al, 1995). These peptides are injected into spinal cord injured adult rat. Regeneration of axons is assayed by immunohistochemistry by comparison with an untreated control. Function improvement is also analyzed.

Aspect 1, Example 6

Screening for candidate factors that can abolish the inhibitory effects of TN-R, MAG, and/or Nogo

Degenerate oligonucleotide sequences are determined for the peptides that can neutralize the inhibitory effects of TN-R, MAG and/or Nogo in vitro and/or in vivo (preferably both). EST databases (preferably databases of CNS-derived ESTs) are searched using the degenerate oligonucleotide sequences. CNS cDNA libraries are screened using labelled degenerate oligonucleotide probes. Hits represent candidate factors

that can abolish the inhibitory effects of TN-R, MAG and/or Nogo. These candidate factors are purified as GST-fusion protein, and the neutralizing effects are tested as described previously for the peptides.

An alternative way to test the neutralizing effects in vivo is to transplant candidate factor-transfected neurons to the injured spinal cord in an experimental animal and observe the neutralizing results.

New factors that can abolish the inhibitory effects of TN-R, MAG and/or Nogo are therefore obtained, and are useful for treating, or in the development of treatments for, CNS damage, especially spinal injury.

Aspect 2, Example 1

Vector Construction

Nucleic acid encoding the following domains was obtained by PCR: N-terminal and 66-Aa extracellular domain of Nogo-A, third to fifth Ig like domain of MAG and the EGF like domain of human TNR.

PCR primers were designed to include restriction enzyme sites and to encode Ala linkers at the ends of the subcloned sequences (see Fig. 1). Therefore, when the PCR products were double digested by restriction enzymes and then connected sequentially, DNA encoding the Ala linkers was located between each pair of neighbouring domains (again, see Fig. 1). The Ala linkers facilitate proper folding of the domains.

Then, the sequentially connected DNA was double digested by BamH I and XbaI and then inserted into a pcDNA 3.1 vector (Invitrogen) to produce a recombinant vector. The recombinant vector was verified by agarose gel and sequence analysis.

Aspect 2, Example 2

Verification of the inhibitory functions of the recombinant vector in vitro

Before the DNA vaccine can be injected into test animals, in vitro testing must be carried out to make sure that the vaccine can be expressed by mammalian cells and can be secreted out of the cell to stimulate the immune system of the host.

To check this, COS-1 cells were transfected with the recombinant vector. After transient expression, the cells and the medium were collected respectively and Western-Blot was performed using antibodies to the inhibitory molecules (Xiao et al, 1996) to verify that the recombinant protein is secreted out the cell and can act as an antigen to stimulate the host immune system when the vector is administered in vivo as a vaccine.

Transfected cells were detected positive for MAG and TNR antibodies compared to non-transfected cells. Immunoreactivity for Nogo was detected in both transfected and non-transfected cells.

48 hours after transient transfection with the recombinant plasmid, the medium was collected and immunoprecipitated

with anti-NogoN or anti-TNR-EGFL antibody using protein A-agarose. The precipitates were separated by 10% and 6% SDS-PAGE and probed for Nogo and TNR, respectively. Each precipitate revealed immunoreactivity for the corresponding antibody, indicating that the protein is being secreted.

Aspect 2, Example 3

Dot-blot of GST fusion proteins with pre-immune sera and anti-sera

Anti-sera were collected from Lewis rats after 2-month vaccination with recombinant plasmid. A serial concentration of GST-Nogo66, GST-NogoN, GST-TNR/EGFL, GST-MAG Ig3-5 and GST were dotted as substrates and subsequently blotted with sera. Anti-sera specially recognized GST fusion proteins, but not GST. Pre-immune sera recognised neither the fusion proteins, nor GST.

Aspect 2, Example 4

Trial of the recombinant vector as a DNA vaccine

To check the regeneration of the injured spinal cord after immunization with the DNA vaccine, the recombinant DNA vector is injected into a test animal. The anti-serum of immunized animals is assayed to verify the immune response after the injection of DNA vaccine.

A semi-transected model for spinal cord injury is used. After a period of time, a morphological study of the injured

spinal cord neuron/axon, and a behavioral study of the animals is performed.

(1) Immunization and spinal cord lesioning

6 week-old female Lewis rats are immunized once weekly with 100µg of recombinant vector from the preceding examples. pcDNA3.1 vector lacking the insertion is used as a control. The vector is injected into the back of the rat. After the rats have produced anti-serum, the spinal cords are lesioned. The rats continue to receive twice monthly immunizations for another 6 weeks.

The rats are anesthetized with Somnitol (1mg/20g body weight), and a lower thoracic laminectomy is done (T9). The dorsal half of the spinal cord is then cut with a pair of microscissors to sever the corticospinal tracts. The depth of each lesion, about 1mm, is estimated by a mark placed on the tip of the micro-scissors. After a 6 week survival period post lesion, the rats are anesthetized and a 5% solution of WGA-HRP (wheat germ agglutinin-horseradish peroxidase) is injected onto the sensory-motor cortex as described (Li et al., 1996). 48 hours after injection of WGA-HRP, the animals are sacrificed by intracardiac perfusion, and the longitudinal cryostat section of the spinal cord is reacted for HRP histochemistry as described (Li et al., 1996).

(2) Verification of the immuno-response of the immunized animals

To verify that the antisera of the immunized animals have the function of blocking the inhibitory effect of the inhibitory domains on neurite outgrowth, a neurite growth

assay is used to test the serum of the immunized animals. A 4-well dish is first coated with solubilized nitrocellulose and preincubated with 5µg/ml poly-L-lysine. The GST fusion inhibitory domains or peptides are placed in the well as a 2µl drop and then incubated for 4 hrs at 37°C. These wells are then incubated overnight at 4°C with the serum from either control mice or mice immunized with the DNA vaccine. The serum is removed, and postnatal day 10 rat cerebellar neurons purified by Percoll density gradient centrifugation are plated at a density of 1 x 10^6 cells per well. The cells are cultured in serum-free chemically defined medium for 24 hrs, fixed with 4% paraformaldehyde, and stained with Coomassie blue. Neurite length is measured using a Universal Image I image analysis system. Data is analyzed using the Student-Newman-Keul's test to determine statistically significant differences (Li et. al., 1996).

(3) Morphological study of the injured spinal cord

To check the regeneration of the transected axon, the rats are perfused with 4% paraformaldehyde, and a 10µm thick longitudinal cryostat section of the spinal cord is picked up on gelatin-coated glass slides. The sections are incubated with a biotinylated goat anti-rat antibody overnight and then with streptavidin-conjugated fluorescein for 1 hr, to detect circulating antibodies.

(4) Functional testing of the animals

The functional recovery of the experimental rat is also carried out. Contact placing response is tested by lightly touching the dorsal aspect of the hind limb without causing joint displacement. The ability of the animals to lift the foot and place it onto the support surface is then assessed

in three to six repetitions. Greater than a 30% response will be scored as positive (Kukel-Bagden et. al., 1993).

(5) Safety testing

The safety of this vaccination approach is assessed by administering the vaccine to test animals (unlesioned and lesioned) and looking for adverse events, especially loss of sensory or motor function and behavioral or cognitive impairments.

CNS tissue is examined histologically to look for autoimmune damage, e.g. by immunostaining using monoclonal anti-CNPase (Sigma C5922, lot 71k4889). CNPase is a protein expressed by oligodendrocytes, 2',3'-cyclic nucleotide 3'-phosphodiesterase.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity and understanding, it will be readily apparent to those of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims.

All documents cited herein are incorporated by reference in their entirety and for all purposes.

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